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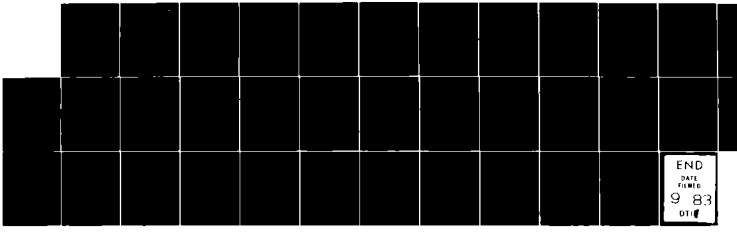

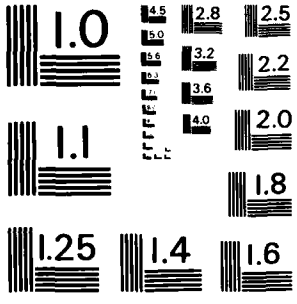

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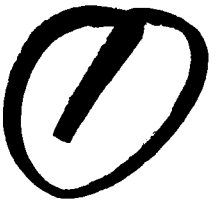

CELLULAR IMMUNE MECHANISMS IN MALARIA

ANNUAL REPORT

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by

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The cell-mediated immune mechanisms responsible for human resistance to malaria need to be better understood. We have examined Thai adults naturally infected with malaria with regard to: 1) the percentages of T, B, Null and Fc receptor bearing cells present during active infection; 2) the functional competence of peripheral blood mononuclear cells as judged by responsiveness to mitogens and cell surface antigens; 3) the ability of serum from infected patients to suppress normal lymphocyte function; and 4) the

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incidence and nature of lymphocytotoxic antibodies in the serum of infected patients.

In comparison to healthy controls, both the percentage and concentration of peripheral T cells were decreased in individuals infected with P. falciparum and P. vivax. The percentage of peripheral B cells was increased but their concentration was unchanged. Both the percentage and concentration of lymphocytes bearing Fc receptors were unchanged in infected individuals. Peripheral blood mononuclear cells from infected patients exhibited normal responsiveness to mitogens and cell surface antigens despite the decrease in T cell number. When sera from infected patients was examined, suppression of cell surface antigen and mitogen induced blastogenesis of normal lymphocytes was observed. Furthermore, sera from such patients contained lymphocytotoxic antibodies. These lymphocytotoxic antibodies were found to be directed primarily against B cells, with the majority having specificity for both T and B cells. In summary: 1) in adult Thai patients naturally infected with malaria, there is a real loss of circulating T lymphocytes with no real change in B or Fc receptor bearing lymphocytes and with no loss of lymphocyte function as judged by mitogenic and antigenic stimulation; 2) in the sera of patients infected with malaria, there are both functional suppressor capabilities and lymphocytotoxic antibodies; and 3) the role of serum immunoregulatory capabilities in modulating the immune response of infected patients may be of importance in determining the chronicity of malaria infection as well as immunologically mediated complications.

Abstract

The cell-mediated immune defense mechanisms responsible for host resistance to malaria are poorly understood. We have therefore examined peripheral blood mononuclear cells and serum from Thai adults naturally infected with malaria in order to determine: 1) the percentages of T, B, Null, and Fc receptor bearing cells present during active infection, 2) the functional competence of peripheral blood mononuclear cells from infected individuals as judged by responsiveness to mitogens and cell surface antigens, 3) the effect of serum from infected patients on normal mononuclear cell function, and 4) the incidence and specificities of lymphocytotoxic antibodies present in the sera of malaria patients. In comparison to healthy controls, both the percentage and concentration of peripheral T cells were decreased in individuals infected with P. falciparum and P.vivax. The percentages of peripheral B cells and Null cells were increased but their concentrations were unchanged. Both the percentage and concentration of lymphocytes bearing Fc receptors were unchanged in infected individuals. Peripheral blood mononuclear cells exhibited normal responsiveness to the mitogens PHA, Con A, PWM, and to allogeneic cell surface antigens despite the decrease in T cell number. Sera from patients infected with malaria inhibited PHA and Con A but not PWM induced mitogenesis by normal cells. The sera also suppressed mixed lymphocyte reactivity by normal lymphocytes. Finally, lymphocytotoxic antibodies were found in the sera of 95% of both P. falciparum and P. vivax infected Thai natives. These antibodies were predominately cold reactive (active at 15^{o} C) but some were active at 37^{o} C. In experiments performed to determine the nature of the cell types against which the lymphocytotoxic antibodies were directed, we found that the antibodies were directed primarily against B cells and in the majority of cases against both T and B cells. Thus, in summary: 1) in adult Thai patients naturally infected with malaria, there

is a real loss of circulating T lymphocytes with no real change in B, Null, or Fc receptor bearing lymphocytes and with no loss of lymphocyte function as judged by mitogenic and antigenic stimulation; 2) sera from infected patients is abnormal in that it suppresses functional capacities of normal lymphocytes; and 3) sera from patients with malaria contain antibodies capable of interacting with B lymphocytes.

Introduction

A better understanding of cell mediated immune mechanisms in malaria may allow more rational approaches to ways of improving immunological defense mechanisms. Therefore, it is hypothesized that the functional characterization of subpopulations of lymphocytes in patients clinically infected with malaria and characterization of the immune modulatory capabilities of serum from these patients will provide insight into the control and effector functions of the cellular immune system's role in host resistance to malaria.

It has been well demonstrated in animals, that circulating antibodies can be passively transferred and will provide protection against malaria (1-3). In addition, the transfer of resistance with cells has been demonstrated for P. berghei in rats (4,5) with a subsequent study that the most likely mechanism by which cell transfer worked was related to antibody production (6). Thus, based on animal studies, lymphocytes are most likely involved in protection against malaria by virtue of their ability to synthesize and secrete antibody and to regulate antibody production. However, the immune response to an active malaria infection in humans is not completely understood.

The changes in lymphocyte populations have been studied in African children infected with P. falciparum (7,8) but it is unclear as to whether these findings can be generalized for other malaria parasites to infected adults living in other areas or what the functional significance of lymphocyte alterations might be. For this reason, we chose to determine the percentages of T, B, and Fc receptor bearing cells and the blastogenic response of unseparated cells to mitogens and cell surface antigens in Thai adults naturally infected with P. falciparum and P. vivax.

In addition, we chose to examine the effect of serum from malaria patients on normal lymphocytes with regard to modulation of mitogen and cell surface antigen induced blastogenesis. These studies were begun on the premise that extrinsic factors as well as intrinsic alterations might be important in regulating the immune

response to malaria.

One of the major control mechanisms in cellular immune function may be lymphocytotoxic antibodies. Antibodies directed against white blood cells were first noted to occur after blood transfusions (9). Subsequent studies revealed that the serum from 17% of multiparous women contained antibodies directed against leukocytes (10). These observations led to the development of the current tissue typing reagents which employ serologically defined (SD) lymphocytotoxic antibodies (11). Further investigations disclosed that both transplantation of allogeneic skin grafts (12) and intradermal injection of allogeneic leukocytes (13) would lead to antibodies directed against white blood cells. All of these above studies, therefore demonstrated the ability of an individual to produce antibodies to histoincompatible cell surface antigens.

More recently, attention has focused on the spontaneous induction of autologous and allogeneic lymphocytotoxic antibodies. The initial studies in this area demonstrated the presence of lymphocytotoxins in patients with infectious mononucleosis, rubella, and measles (14). Of particular interest was that these antibodies were optimally detected at 15^{o} C. Shortly thereafter, similar antibodies were detected in systemic lupus erythematosus (15). Subsequently, a variety of other disease states have been shown to be associated with an increased incidence of lymphocytotoxic antibodies, including inflammatory bowel disease (16) and multiple sclerosis (17). We therefore also decided to investigate sera of adult Thai patients naturally infected with either P. falciparum or P. vivax for warm and cold reactive lymphocytotoxic antibodies and to characterize the types of cells against which these antibodies are directed.

Methods

Isolation of mononuclear leukocytes:

Peripheral blood mononuclear leukocytes (MNL) were obtained according to the method of Boyum (18). Heparinized blood was drawn from each malaria patient immediately before treatment or 14 days after the initiation of treatment. The blood was diluted 1:2 in Hanks Balanced Salt Solution (HBSS, Grand Island Biological Co. (GIBCO) New York) and layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, New Jersey). Following centrifugation, the MNL were removed by Pasteur pipette from the interface. The MNL were then adjusted by hemocytometer count to a concentration of 2×10^6 cells in HBSS.

Preparation of sheep red blood cells for rosette forming assays:

Sheep red blood cells (SRBC) in Alsever's solution were filtered with sterile gauze and washed with thriethanolamine-buffered salt solution (TBS). The SRBC were resuspended at a concentration of $7\text{-}8 \times 10^9$ cells/ml in TBS containing 0.1% gelatin (Baltimore Biological Laboratory, Baltimore, Maryland). Unmodified SRBC (E rosettes) were used to determine the percentage of T cells. In order to detect cells with Fc receptors, SRBC were coated with subagglutinating amounts of 7S (IgG) anti-SRBC (Cordis Laboratories, Miami, Florida) to form EA rosettes. In order to detect cells bearing complement receptors, SRBC were coated with subagglutinating amounts of 19S (IgM) anti-SRBC (Cordis) and then incubated with fresh mouse serum.

Procedures for detecting rosette forming lymphocytes:

The methods of Mendes et al were employed with modifications as previously described (19). The percentage of cells forming E rosettes were determined after 5 minutes, 1 hour, and 18 hours (overnight) at 4° C. The percentage of cells forming EA and EAC rosettes was determined after 30 minutes at 37° C. In calculating the percentages of lymphocytes forming rosettes with three or more red cells, both sides of a hemocytometer chamber were counted and the values

of rosetting and nonrosetting lymphocytes were averaged.

Direct fluoresceinated antibody technique:

Surface immunoglobulin bearing B lymphocytes were also identified by staining with fluorescein labelled anti-human-immunoglobulin (GIBCO) . The methodology of Chess et al (19) was employed with modification. Briefly, 3-4 x 10^6 MNL were incubated for 1 hour at 37^o C to remove non-specifically adsorbed immunoglobulin (20), washed three times, centrifuged, and fluoresceinated anti-human immunoglobulin added. After mixing and incubation, the cells were again centrifuged and washed in HBSS containing 10% heat inactivated fetal calf serum (GIBCO). After final centrifugation, the cells were resuspended in cold glycerol phosphate buffered saline and the percentage of fluorescing cells determined using a Leitz ortholux microscope equipped with BG 12 and K 530 filters with a 220 watt high pressure mercury lamp. As with the rosette assays, counts were done blind with the sample origin unknown, readings were done in duplicate, and the results were averaged.

Separation of mononuclear cells into subpopulations:

Isolation of human lymphocyte subpopulations were carried out using current modifications as described below of our previously published methods (19,21-23). In brief, monocytes were removed from mononuclear cells by passage over a G-10 column. The resultant monocyte depleted lymphocyte population was incubated at 37^o C for one hour to remove passively adsorbed immunoglobulin from the Null cells and then passed over a Sephadex-G-200 anti $(Fab')_2$ column to separate the cells into a non-immunoglobulin bearing (T plus Null) cell population and an immunoglobulin bearing (B) cell population. The T plus Null cell populations were fractionated using sheep red blood cell (E) rosette formation followed by keeping the rosettes at 4^o C overnight and then by Ficoll-Hypaque gradient sedimentation. The non-E rosette forming lymphocytes (Null cells) at the interface were then harvested. The E rosette forming T cells in the pellet were obtained by distilled

water lysis of the sheep RBC's. Fc bearing cells in the T cell population were then removed by formation of rosettes with chicken RBC's coated with IgG-anti-CRBC antibody followed by Ficoll-Hypaque density sedimentation. The interface contains purified T cells and the Fc bearing T cells in the pellet can be recovered by distilled water lysis. Residual T cells in the B cell population were removed by overnight E rosette formation followed by Ficoll-Hypaque sedimentation. Using these separation techniques, we start with 500 cc of normal blood from which 600 to 800 million mononuclear cells are obtained. We have found that macrophage depletion must be done prior to separation in order to avoid excessive cell loss when isolated subpopulations are macrophage depleted. The G-10 macrophage depletion technique results in a 40% to 55% cell loss and a lymphocyte preparation which has less than 0.1% monocytes by myeloperoxidase stain. The resultant 300-400 million lymphocytes are passed over the anti-$(Fab')_2$ immunoabsorbent column resulting in another 15% non-specific loss of cells. The immunoglobulin bearing (B) cell enriched population contains 2% to 15% E rosetting cells which are removed by E rosette depletion with a 35% to 50% cell loss. Of importance is that prior to passage over the anti-$(Fab')_2$ column the unseparated lymphocytes are washed well at 37^0 C in order to remove nonspecifically absorbed immunoglobulin which would cause the L cells of Horowitz and Lobo (20) to adhere to the column. This step is necessary to place the L cells (which are most likely similar to K or "Null" cells) in the non-immunoglobulin bearing (T plus Null population). Thus T plus Null cell populations are also fractionated by a series of rosette techniques each of which results in a 35%-50% cell loss. The sequence of fractionation procedures starting with 600 to 800 million mononuclear cells results in: 1) 100 to 150 million T cells of which 78% to 86% form E rosettes and less than 0.5% bear surface immunoglobulin or bear Fc receptors (EA rosette forming); 2) 5 to 7 million Fc receptor bearing T cells of which 70%

to 82% form E rosettes and 56% to 68% form EA rosettes but less than 0.5% bear surface immunoglobulin; 3) 10 to 16 million non-T, non-B cells (Null, K or L cells) of which < 0.5% form E rosettes, < 0.5% bear surface immunoglobulin and 67% to 82% bear Fc receptors; and 4) 15 to 30 million B cells of which less than 1% form E rosettes and 84% to 96% bear surface immunoglobulin.

Mitogen and cell-surface antigen induced blastogenesis:

The proliferative response of peripheral blood lymphocytes to mitogens was investigated by standard techniques as we have previously described (21-23). In brief, 1 x 10^5 cells for mitogen studies were placed in triplicate in microtiter plates with either media, phytohemagluttinin, concanavalin A or pokeweed mitogen. Cultures were pulsed after 4 days with 0.05 ml of media containing 0.2 uCi methyl-^{3}H-thymidine. After 4 hours in culture with the thymidine, the cells were harvested using a MASH II extractor. One-way mixed lymphocyte cultures (MLC) were performed as previously described (21,23). In brief, 2 x 10^5 responding cells and 2 x 10^5 mitomycin-C treated stimulating cells in 0.2 ml of final culture medium were placed in triplicate in microtiter plates. After 6 days, the cultures were pulsed with 0.2 uCi of ^{3}H-thymidine for 16 hours and harvested with a MASH II apparatus.

Serum studies:

Individual serum samples were obtained from 58 Thai adults naturally infected with P. falciparum and 50 with P. vivax. Sera was also obtained from 37 normal healthy individuals living in the same region as the infected patients. None of the infected or control individuals had a history of receiving blood transfusions and none were on medications. To further reduce the possibility that any lymphocytotoxic antibodies detected might be due to causes other than malaria infection, only male patients or females with no more than a single pregnancy were accepted into the study. The degree of parisitemia was assessed by a Giemsa stained peripheral blood smear. After the blood was clotted at room

temperature, the serum was removed and stored at -20° C. The patients participating in this study were then treated by the staff of the National Malaria Eradication Project of Thailand.

Lymphocytotoxic antibody assay:

Cytotoxic assays were run in duplicate in flat bottomed tissue culture trays (Costar Cluster 96, Cambridge, Massachusetts). The methodology was modified from previously described techniques (15). In brief, peripheral blood mononuclear cells (PBC) were isolated according to the method of Boyum (18). After three washes in Hanks Balanced Salt Solution (HBSS), the cells were adjusted to a final concentration of 3×10^6/ml in HBSS containing 10% heat inactivated fetal calf serum and 0.1 ml added to each well of the culture tray. Subsequently, at either 15° C or 37° C, 0.1 ml of each sera (in duplicate) was added to appropriate wells for 30 minutes followed by addition of 0.1 ml of fresh rabbir serum as a source of complement. After an additional 4 hours at either 15° C or 37° C, the percentage of dead cells was determined by eosin dye inclusion. Different normal healthy lab personnel served as donors of the PBC's used as indicator target cells for the lymphocytotoxic antibodies. Both control sera and patient sera were run simultaneously. Sera from patients with systemic lupus erythematosus served as positive controls. Reproducibility of the assay (checked by repeating the same sera directed against the same targets at different times) demonstrated essentially identical results for the sera tested.

Serum inhibitory studies:

The effect of serum from infected patients on mitogen and cell surface antigen induced blastogenesis by normal lymphocytes was assessed by placing individual (20%) or pooled sera (20%) from patients in the mitogenesis assays described above using normal PBC instead of patient PBC.

Treatment of patients:

The treatment of malaria patients participating in these studies was

administered by the staff of the National Malaria Eradication Project of Thailand. Patients infected with P. falciparum were given 1 gram of sulfadoxine (Fansidar) and 50 mg pyrimethamine in a single dose and 15 mg primaquine/day for 4 days. Patients with P. vivax infections were treated with 1500 mg chloroquine and 15 mg primaquine/day for 5 days.

Statistical analysis:

The Student's t test was used and $p < .01$ was considered necessary to obtain "significance".

Results

Lymphocyte subpopulation enumeration and functional characterization:

Forty-nine patients infected with either P. falciparum or P. vivax were studied. The 24 P. falciparum patients ranged in age from 17 to 48 years (mean 25). Twenty-five patients with P. vivax were studied, who ranged in age from 16 to 45 years (mean 24). A group of 21 healthy Thais with no previous history of malaria served as controls. These individuals ranged in age from 19 to 45 years (mean 32) and were of similar ethnic background and geographic area as the study group.

Circulating lymphocyte subpopulations were studied by E, EA and EAC rosette fomation and fluorescein labelled anti-human-immunoglobulin (FITC) staining and the results expressed either as a percentage (Table 1) or as an absolute concentration (Table 2). To determine the percentage and concentration of T lymphocytes, the E rosette technique was employed with incubation times of 5 minutes, 1 hour and 18 hours. The 5 minute values indicated that there was a marked suppression in the percentage (Table 1) and concentration (Table 2) of T cells in both P. falciparum and P. vivax patients. There was likewise a reproducible pattern of a decreased percent and concentration of E rosette forming cells (T lymphocytes) in patients with malaria, at assay times of 1 hour and 18 hours (Tables 1 and 2). The decrease in percent and concentration of E rosette forming lymphocytes (T cells) were statistically significant in 10 of the 12 comparisons (Tables 1 and 2).

The EA rosette technique was utilized in evaluating the percentage of circulating Fc bearing cells. Patient cells showed virtually identical mean values in comparison with normal controls and no significant change in terms of comparison with normal controls and no significant change in terms of either percentage (Table 1) or concentration (Table 2) of Fc bearing cells was observed. Application was made of the EAC rosetting technique and the FITC staining

technique in identifying circulating B lymphocytes. The mean EAC values were 25% for patients with falciparum malaria as compared with 24% for the vivax patients and 16% for the normal controls. The FITC cell values for patients were likewise elevated and showed excellent correlation with those for the EAC (B cell) rosettes with mean percentages of 24 for falciparum patients, 22 for vivax patients and 15 for the normal group. The elevated percentages of B cells in the patients compared to controls was statistically significant ($p < 0.01$) for both techniques. However, when the concentration of B cells was calculated, there was essentially no difference found in the number of B cells in patients with malaria in comparison to normal controls (Table 2).

Examination of Patient Peripheral Blood Mononuclear Cells in Assays of Blastogenic Responsiveness to Mitogenic Lectins and Allogeneic Cell Surface Antigens:

We next examined the ability of peripheral blood mononuclear cells (MNC) from patients infected with P. falciparum or P. vivax to respond to selected mitogens in culture. The responsiveness of patient peripheral blood MNC to Con A, PHA, and PWM is summarized in Table 3. The responsiveness of the patient cells was equal to that of normal controls. Thus, with regard to stimulation by mitogenic lectins, peripheral blood MNC from mildly ill patients naturally infected with malaria did not show a decreased responsiveness.

We then turned our attention to the allogenic mixed leukocyte reaction (MLR) in order to examine the capacity of infected patient's MNC to respond to or stimulate allogeneic cells from individual normal volunteers. As can be seen in Table 4, when cells from patients with P. falciparum were used as responders in MLR, a normal response to cell surface antigens on allogeneic normal cells was observed. In contrast, MNC from P. vivax patients had a statistically significant decreased responsiveness ($p < .05$) in allogeneic MLR. When cells from patients with malaria were used as stimulators in the MLR, individual normal responding cells exhibited significantly ($p < .05$) decreased blast

transformation. Thus, although MNC from malaria patients functioned normally in response to mitogens, P. vivax MNC were abnormal as responders in MLR and both P. vivax and P. falciparum MNC were suboptimal in their capabilities to function as stimulators in the one-way allogeneic MLR.

Modulation of Normal Mononuclear Cell Function by Patient Sera:

We next examined the effects of sera from patients with malaria on mitogenic responsiveness and on the MLR using normal human MNC from single individuals as the indicator cells in both systems. As can be seen in Table 5, in experiments using 20% pooled sera from patients with P. falciparum or P. vivax, the mitogenic responsiveness of normal peripheral blocd mononuclear cells was markedly reduced to both PHA and Con A. There was no statistically significant decrease in the mitogenic responsiveness to PWM. We also investigated the effect of individual sera from patients with malaria on responsiveness to the mitogens and the results were similar to that seen with the pooled sera.

Finally, we studied the effect of 20% pooled sera from P. falciparum patients on normal allogeneic cells in MLR. These results are displayed in Table 6. The patient sera decreased the stimulation index from 9.9 to 5.1. Thus, sera from patients with P. falciparum appeared to have an inhibitory effect on the normal blastogenic response to allogeneic cell surface antigens in vitro.

Lymphocytotoxic Antibodies in Sera from Patients:

Sera from 58 patients with P. falciparum, 50 patients with P. vivax, and 37 normal healthy controls were examined for the presence of lymphocytotoxic antibodies in assays run at 15^{o} C and 37^{o} C. There was a wide range of lymphocytotoxic activity exhibited by the individual sera, causing from 2% to 78% of the target mononuclear cells to be lysed (Figure 1). As seen in Table 7, the killing due to control sera averaged only 3.2% (15^{o} C) and 3.3% (37^{o} C). None of the control sera lysed more than 5% of the indicator cells (Figure 1). When sera from patients with either P. falciparum or P. vivax were examined, marked lymphocytotoxic

activity was observed (Figure 1, Table 7). If the mean plus two standard deviations of the control value is used as an upper limit of normal in studies done at 15° C, 98% of P. vivax patients and 95% of P. falciparum patients had antibodies in their sera which were cytotoxic for a greater percentage of indicator lymphocytes than would normally be expected (Figure 1). The average percent of indicator cells was significantly greater for serum from patients with vivax and falciparum malaria than controls ($p < .001$; Table 7). The lymphocytotoxic antibodies were significantly more reactive using 15° C as the incubation temperature than using 37° C (Figure 1, Table 7, $p < .001$). Furthermore, this temperature dependent aspect held for individual sera investigated, in that the sera which exhibited greater reactivity at 15° C also exhibited greater reactivity at 37° C. There was no relationship between the percent of indicator mononuclear cells killed and the patient's degree of parasitemia, age or sex.

In addition, we have examined the cell types which the lymphocytotoxic antibodies are directed against (Table 8). The antibodies were primarily directed against B cells, with the majority having lymphocytotoxic activity against both T and B cells.

Discussion

We have observed that Thai adults naturally infected with either P. falciparum or P. vivax have a decrease in the percentage and concentration of T lymphocytes, an increase in the percentage but no change in the concentration of B lymphocytes and no change in either the percentage or concentration of Fc receptor bearing cells. However, T cell function, as judged by lymphocyte responsiveness to mitogenic lectins and to cell surface antigens appears to be intact despite the decrease in T cell number and concentration.

These findings are in agreement and disagreement with various aspects of previously reported results by Wyler (7), who studied 30 children and 3 adults in West Africa with P. falciparum, and Ade-Serrano and Osunkoyo (8), who also studied children with P. falciparum. Both previous studies (7,8) and the present study found that during acute infection with malaria, the percentage of cells forming E rosettes (T cells) was decreased. Both the study of Wyler (7) and the present study also found a decrease in the concentration of T cells, thus signifying a true loss of T cells. Both previous studies (7,8) found similar results to the present study in observing an increase in the percentage of B cells using EAC rosettes. More importantly Wyler (7) found, as we did, that there was no increase in the concentration of B cells.

While the T cell depression observed in infected patients could represent a transient relocation of lymphocyte pools, it would be reasonable to hypothesize that the loss of T cells could be due to destruction of a specific T cell subclass. Furthermore, the ability of T cells or Fc receptor bearing cells to participate in cellular cytotoxic reactions might be enhanced or depressed during active infection due to the changes in cell populations. Whatever the underlying mechanism(s), the depression in numbers of T lymphocytes may not be limited to circulating cell populations but may involve T cell depletion of the lymphoid organs as well. Indeed, Krettli and Nussenzweig reported

depletions in mouse lymphocyte populations in thymus glands and lymph nodes during infection with P. berghei (24).

Following the classification of the changes occurring in relative proportions of lymphocyte subpopulations as observed above, we then studied the functional competence of the patient's lymphocytes. In particular, the loss of T cells in the patients raised the question of which T cell functions might be lost.

In our experiments, we observed that peripheral blood mononuclear cells (MNC) from Thai adults infected with either P. falciparum or P. vivax malaria exhibited normal responsiveness to the mitogenic lectins concanavalin A (Con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM). It thus appears that, despite the loss of T cells in the peripheral blood of patients with malaria, the functional capabilities of the remaining MNC as assessed by mitogen induced blastogenic responsiveness is essentially intact. The major cell which responds to Con A and PHA is the T cell (21-23); however, both B cells and Null cells are also capable of responding to PHA and Con A as long as 5-20% T cells are also present. There are several possibilities to explain the normal MNC responsiveness to Con A and PHA in malaria patients with an apparent loss of circulating T cells: 1) B and Null cells, as well as the remaining T cells, may be the cells responding to these lectins, since at least 30% of the peripheral blood MNC in patients with malaria are T cells, 2) T cells which were lost during the acute infection may not be the cells which would normally respond to mitogenic lectins and thus the population of cells remaining could respond normally, and 3) even though the responsiveness of MNC from patients with malaria to mitogens is intact, their functional capabilities in other assays (cell mediated cytotoxicity, antibody synthesis) may be deficient.

In the allogeneic mixed leukocyte reaction (MLR) the principle responding cell is the T cell, while macrophages, Null cells and B cells function as

stimulators (21-23). The normal degree of stimulation in the MLR is much less than that observed in lectin induced mitogenesis, because a smaller percentage of cells proliferate in response to allogeneic cell surface antigens than proliferate in response to nonspecific mitogenic lectin stimulation. Sixty to seventy percent of the cells will become blasts when cultured with the mitogenic lectins, whereas only 2-5% of the cells become blasts in MLR. Thus, mixed leukocyte culture reactivity is potentially a more subtle test of the capacity of a discrete number of cells to specifically recognize and be stimulated by allogeneic cell surface antigens. Furthermore, in the MLR Null cells, B cells, and macrophages do not function as responders (21-23). In our present experiments, no loss of MLR cellular responsiveness was observed with P. falciparum cells, while P. vivax cells functioned poorly. It is conceivable that T cells which are lost in P. falciparum patients are non-mixed leukocyte culture responsive T cells, while those lost with P. vivax infection may be T cells which normally respond in the MLR. Alternatively, it is possible that even though the reactivity in MLR is normal with P. falciparum cells, other specific T cell functions might be impaired.

Despite normal responsiveness in the MLR, peripheral blood MNC from patients with P. falciparum did not exhibit normal stimulatory capabilities. Cells from P. vivax patients also had decreased stimulating capabilities. Because the percentage and concentration of Null cells, B cells, and macrophages are not decreased in patients with malaria it must be assumed that there is a functional stimulatory impairment despite the cells being present.

Thus, with regard to the allogeneic, one-way, MLR by patient MNC, we have observed several unexpected dichotomies: 1) normal responsiveness with P. falciparum MNC despite the apparent loss of the responding cell type (as suggested in our work cited above) and 2) decreased stimulatory capabilities despite the presence of the appropriate cells in both P. falciparum and P. vivax.

One possible explanation for these results is that serum factors in patients may influence cell function. Indeed we found in the course of our experiments that pooled sera from both P. falciparum and P. vivax patients induced marked inhibition of normal peripheral blood MNC responsiveness to PHA and Con A. In contrast, inhibition of responsiveness to PWM was not observed. Pooled sera from P. falciparum patients also inhibited responsiveness of normal cells in the MLR. We have also observed that sera from malaria patients contain cold reactive (15^{o} C) and warm reactive (37^{o} C) lymphocytotoxic antibodies. Because malaria sera contain antibodies capable of reacting with normal peripheral blood MNC, it is possible that these sera modulate cell function through lymphocytotoxic activity and produce the inhibition of allogeneic MLR and mitogen induced proliferation. The decrease in allogeneic MLR could be due to either a responding cell or stimulating cell defect. We have not yet determined, however, against what cell types the lymphocytotoxic antibodies are directed. Furthermore, although we have shown that sera from patients with malaria will inhibit responsiveness to PHA, Con A, and allogeneic cell surface antigens, we have not established that lymphocytotoxic antibodies are involved in mediating the suppression.

It should be noted that nonspecific assays such as mitogenic responsiveness or the MLR may not represent functions directly involved in the host response to the malaria parasites. Therefore, effector functions such as cell mediated cytotoxicity, phagocytosis, and antibody synthesis may be more relevant and thus will be of great interest for future studies. If the capability of malaria sera to modulate the host immune response is of importance, defense mechanisms which are more likely to be involved in host protection should be examined in future studies.

There is evidence from previous studies of other investigators, that active malaria interfers with a normal immune response. First of all, in mice infected

with malaria, there is suppressed antibody production, reduced severity of autoimmune disease, and increased susceptibility to tumor viruses (25). Secondly, there is the immunopathologic effect of antigen-antibody complexes causing nephrotic disease in young children with P. malariae (26,27). Thirdly, anemia due to increased phagocytosis of uninfected erythrocytes has been noted in animals, yet the mechanism of this increased destruction is unknown. In this regard, although antibodies to red blood cells have been found with increased frequency in patients with malaria, it is possible that they may be secondary to the fragmented red cells rather than causative of an autoimmune hemolytic process (25,27). Finally, increased levels of auto-antibodies directed against heart, thyroid, and gastric parietal cells associated with elevated IgM levels and high titers of malarial antibodies have been noted in immigrant and indigenous peoples in Uganda (28). Thus, infection of a host with malaria has been shown to result in a variety of adverse effects on and of the immune system. Our studies have demonstrated an additional immunologic abnormality: antibodies directed against peripheral blood mononuclear cells in general, and B cells in particular.

Initial studies of lymphocytotoxic antibodies demonstrated their presence in a variety of clinical syndromes, with viral and presumed autoimmune diseases predominating. A parasitic infestation, malaria, can can now be included in the list of diseases which induce lymphocytotoxic antibody formation. There are several possible ways in which infection with malaria might induce the production of lymphocytotoxic antibodies. Firstly, the antibodies might actually be directed against malaria antigens and just be cross reactive against lymphocytes. Secondly, malaria antigens could have become bound to the lymphocytes, causing the antibodies to be formed against altered self antigens. Thirdly, the process could be due to the presence of an immunologic stimulus which nonspecifically induced lymphocytotoxic production. Fourthly, the antibodies could be an

important part of the protective host response to malaria by causing a deletion of suppressor cells in order to enable heightened cytotoxic or antibody effector functions. Future in vivo and in vitro studies will be needed in order to answer which of these possible explanations is correct.

The target cell of the lymphocytotoxic antibodies in sera from malaria patients is predominantly the B cell, with the majority of antibodies having both T and B cell reactivity. Previous studies indicate that cold reactive lymphocytotoxic antibodies are predominantly IgM and directed against T cells (29,30) in disease states. In this regard, it should be noted that cold reactive IgM lymphocytotoxic antibodies directed against B cells occur in approximately 20% of normal individuals (31). The lymphocytotoxic antibodies in malaria patients are in most instances directed against more than B cells as shown by our initial observation that greater than 12% of target cells are killed by the majority of sera studied and by the definitive finding that almost half of the sera have T cell reactivity. Nevertheless, future studies will be needed to address the questions of the type of lymphocytotoxic antibodies found in malaria patients and their relationship to disease activity.

The question remains as to the in vivo relationship of these lymphocytotoxic antibodies and the decreased T cell numbers found in the peripheral blood of actively infected patients. Although the antibodies are cytotoxic in vitro only at 15^{o} C, it is possible that they function in vivo similarly to antibodies in cold agglutinin disease. That is, in cold agglutinin disease IgM antibodies fix to red blood cell (RBC) in the cooler peripheral parts of the body, fix complement, and then elute off the RBC in the warmer parts of the body leaving a complement coated RBC to be phagocytosed at a later time (32). Furthermore, antibodies from different individuals with cold agglutinin disease vary as to the optimal temperature at which they act and thus the severity of the disease process appears to depend upon the characteristics of the particular

individual antibody (32). Therefore, there could possibly be a variable effect of the lymphocytotoxic antibodies in patients with malaria depending on the temperature at which each person's antibody is optimally functional. It is important, of course, to consider the other alternatives as to the relationship between the lymphocytotoxic antibodies and reduced numbers of T cells: 1) The reduced T cells could actually have led to lymphocytotoxic antibody production through loss of a suppressor cell regulatory effect, or 2) there may be no relationship between the two and the T cell loss is actually due to other factors. Additional studies will be necessary in this area as well in order to determine the in vivo relevance of lymphocytotoxic antibodies in patients with malaria.

A continued better understanding of the role of the immune system in the human host response to infection with malaria will hopefully be achieved by future studies using patients with malaria on:the cytotoxic capabilities of peripheral blood mononuclear cells; cell mediated and serum mediated immunoregulatory abnormalities; the nature and capabilities of lymphocytotoxic antibodies; and the characteristics of in vitro antibody synthesis and secretion by peripheral blood mononuclear cells. As our understanding of the normal human immune response to malaria infection grows, our ability to devise methods for augmenting existing or devising new defense mechanisms will also progress.

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Table 1

Changes in the Relative Percentages of Peripheral Blood Lymphocyte Subpopulations in Thai Adults with *P. falciparum* and *P. vivax*

	Percent of Peripheral Blood Lymphocytes Identified by					
	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes	EA rosettes	EAC rosettes	Surface Immunoglobulins
Controls (N = 21)[a]	41 ± .9[b]	54 ± .9	63 ± .3	5 ± .3	15 ± .2	15 ± .5
P. falciparum (N = 24)	28 ± 1.0	41 ± 1.0	52 ± .5	5 ± .5	25 ± .5	24 ± .6
	($p < .001$)[c]	($p < .001$)	($p < .001$)	($p < .9$)	($p < .001$)	($p < .001$)
P. vivax (N = 25)	29 ± 1.0	41 ± 1.0	54 ± .7	5 ± .3	24 ± .5	22 ± .5
	($p < .001$)	($p < .001$)	($p < .001$)	($p < .9$)	($p < .001$)	($p < .001$)

a N = Number of subjects studied.

b Mean ± S.E.M.

c p value for malaria infected group in comparison to control

Table 2

Changes in the Concentration of Peripheral Blood Lymphocyte Subpopulations in Thai Adults with *P. falciparum* and *P. vivax*

	Number of Peripheral Blood Lymphocytes per mm^3 Identified by					
	5' E rosettes	1 hr. E rosettes	18 hr. E rosettes	EA rosettes	EAC rosettes	Surface Immunoglobulin
Controls [a] (N = 21)	1162 ± 475 [b]	1486 ± 154	1750 ± 136	137 ± 17	447 ± 35	400 ± 36
P. falciparum (N = 24)	564 ± 56	781 ± 69	999 ± 85	100 ± 13	486 ± 38	467 ± 34
	($p < .02$) [c]	($p < .001$)	($p < .001$)	($p < .1$)	($p < .5$)	($p < .2$)
P. vivax (N = 25)	557 ± 46	1086 ± 311	1058 ± 96	97 ± 10	483 ± 37	429 ± 33
	($p < .01$)	($p < .3$)	($p < .001$)	($p < .05$)	($p < .5$)	($p < .5$)

a N = Number of subjects studied

b Mean ± S.E.M.

c p value for malaria infected group in comparison to control

Table 3

Responsiveness of Peripheral Blood Mononuclear Cells

from Normals and Malaria Patients to Mitogens

	Con A		PHA		PWM	
	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.
P. Vivax (9)	27,068 ± 2,511[a]	41 ± 9	65,620 ± 3,758	91 ± 15	31,853 ± 3,228	49 ± 12
Controls (9)	28,993 ± 2,603	39 ± 4	61,471 ± 4,457	89 ± 16	29,349 ± 4,954	33 ± 5
P. Falciparum (6)	23,562 ± 2,001	34 ± 5	53,957 ± 3,279	74 ± 6	26,092 ± 2,333	37 ± 4
Controls (6)	28,711 ± 1,962	49 ± 7	57,537 ± 3,127	100 ± 17	27,508 ± 2,528	49 ± 10

[a] Mean stimulation index ± standard error of mean for number of experiments in parenthesis.

Table 4

Responsiveness of peripheral blood mononuclear cells from normal individuals and malaria patients in one way mixed lymphocyte cultures[a]

Patients infected with *P. vivax* (22):	Normal responders	Patient responders
Normal stimulators	8.5 ± 1.6	5.2 ± 0.9
Patient stimulators	5.9 ± 0.9	N.D.[b]
Patients infected with *P. falciparum* (22):		
Normal stimulators	11.3 ± 2.2	12.2 ± 3.2
Patient stimulators	5.1 ± 1.0	N.D.

[a] Individual responder and individual stimulator cells were incubated 120 hours at 37° C, pulsed an additional 24 hours with ^{3}H-thymidine, and stimulation index determined. Results expressed as mean stimulation ± S.E.M. for number of experiments (each a separate patient) performed (in parentheses).

[b] Not done.

Table 5

Effect of Pooled Sera from Patients (10 *P. vivax* and 10 *P. Falciparum* Patients) on Responsiveness of Normal Peripheral Blood Mononuclear Cells to Mitogens

Pooled Sera	Phytohemagglutinin	Concanavalin A	Pokeweed Mitogen
P. vivax (15)	81 ± 9[a]	53 ± 10	74 ± 13
Normal Controls (15)	160 ±15	127 ± 20	105 ± 23
Statistical Significance	$p < .001$[b]	$p < .01$[b]	$p < .3$
P. falciparum (16)	81 ± 9	69 ± 9	86 ± 12
Normal Controls (16)	157 ± 9	112 ±11	81 ± 14
Statistical Significance	$p < .001$[b]	$p < .01$[b]	$p < .9$

[a] Mean stimulation index ± standard error of the mean for the number of individual experiments in parenthesis.

[b] Statistically significant difference.

Table 6

Effect of Pooled Sera From 10 *P. falciparum* Patients on Responsiveness of Normal Peripheral Blood Mononuclear Cells in the Allogeneic Mixed Leukocyte Reaction (MLR)

Pooled Sera	Stimulation Index of MLR
Normal (44)	9.9 ± 1.4[a]
P. falciparum (12)	5.1 ± .6
Statistical Significance	p .01[b]

[a] Mean stimulation index ± standard error of the mean for the number of experiments in parenthesis.

[b] Statistically significant difference.

Table 7

Lymphocytotoxic Antibodies in Sera of Thai Adults Infected with Malaria

Sera	Percent Peripheral Blood Cells Killed	
	15° C	37° C
Normal Control (37)[a]	3.2 ± 0.1[b]	3.3 ± 0.1
P. falciparum (50)	19.9 ± 1.9	7.0 ± 0.7
P. vivax (58)	28.7 ± 3.3	8.5 ± 0.1

a: Number of individual sera examined in parenthesis

b: Mean ± 1 SEM

Table 8

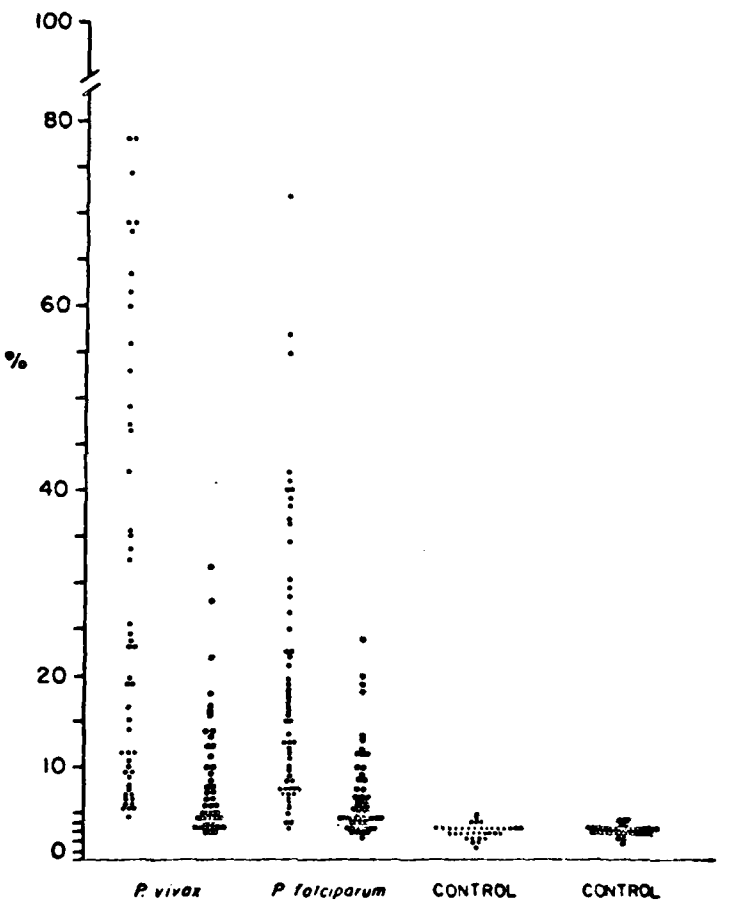
Cell Types Against Which Lymphocytotoxic Antibodies in Sera of Patients with Malaria are Directed

Sera	T Cells Only	B Cells Only	Both T and B Cells	No Cells
Normal control sera (16)[a]	0	0	0	100%
P. falciparum sera (31)	3%[b]	29%	48%	19%
P. vivax sera (25)	12%	24%	44%	16%

[a] : number of sera examined

[b] : percent of sera examined, which had lymphocytotoxic antibodies capable of killing T cells only, B cells only, both T and B cells, or no cell types.

Figure 1



lymphocytotoxic antibodies in sera of Thai adults infected with malaria. Individual sera from patients with P. falciparum, P. vivax, and normal controls were examined at either 15° C or 37° C.

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